

Precipitation of Zn₂Al LDH by urease enzyme

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A biomineralization process based on the promotion of precipitating agent by the urea-urease enzymatic system is developed to prepare ZnAl layered double hydroxide materials and the effects of the enzymatic reaction parameters on the structural and textural properties of the materials are investigated on the basis of XRD and EM analysis.

Biomineralization¹ is a natural process in which living organisms build their own multiscale structure, from the atomic to the macroscopic level, via the precipitation of inorganic minerals at the surface of biomembranes or cells. This leads to hierarchical architectures with exceptional mechanical or physico-chemical properties. This is the case of the silica walls of diatom algae. One of the most exciting challenges of materials chemistry is currently to mimic such natural processes in order to prepare materials with controlled structure and morphological properties. Thus a novel strategy of materials synthesis can be profitably developed on the understanding of biomineralization used by living organisms. This approach is based on the progress made in soft chemistry synthesis at the interface of inorganic and organic or bioorganic materials. In such conditions, precipitation of the synthetic mineral occurs at the surface of the organic matter and is directed by the coulombic interactions between metallic precursors and derived inorganic nuclei and the organic microstructures such as microorganisms or bio-macromolecules. On the other hand, fundamental understanding at the molecular level of the physical and chemical associations between these inorganic structures and cells or biopolymers will help to better understand the adsorption of biomolecules or organisms at the surface of mineral and to develop novel biomaterials based on enhanced nanocomposite associations. Moreover, these associations can involve specific properties of both partners leading to a novel generation of intelligent materials with multifunctionalities or high sensitivity.

Owing to their structure and morphology, natural or synthetic inorganic layered solids such as clays or layered hydroxides display extended surfaces and surface electrical potentials opened for interface reactions. They are known to develop strong affinities for organic polymerised matter leading for example to the formation of stable organo-clay complexes with a key role in soil structuration. These layered solids appear to be suitable minerals in terms of structure and surface properties to grow at the surface of organic microstructures and to form novel hybrid or

nanocomposite materials. Recently Stucky *et al.*² evidenced the biomineralization and the structural and mechanical role of a layered mineral, atacamite Cu₂(OH)₃Cl, in the jaws of the marine bloodworm *Glycera dibranchiata*. Biogenesis of green rust has also been proven to be generated by many microorganisms and particularly by *Shewanella putrefaciens*, a bacteria widely developed in natural ecosystems. Green rust minerals are Fe^{II}-Fe^{III} layered hydroxysalts involved in the soil redox processes in anaerobic conditions.³ They display a key role in denitrification reactions or degradation of pollutants.⁴

Analogous materials are layered double hydroxides (LDHs),⁵ also called anionic clays built on a stacking of positively charged octahedral sheets with [M^{II}_{1-x}M^{III}_x(OH)₂]^{x+} composition (M^{II} and M^{III} are divalent and trivalent metal ions respectively) able to intercalate anionic guest species [X_{nm}^{m-}·nH₂O]^{x-} in the interlayer domains. The LDH compounds abbreviated as [M^{II}-M^{III}-X] are characterized by the high charge density of the sheets, modulated by the M^{II} : M^{III} ratio. The hydrated anions in the interlayer spaces can be replaced with almost any desired organic or inorganic anion, by utilizing simple ion exchange methods.⁶ From a fundamental point of view, LDHs are very attractive as structural models of bidimensional materials, displaying very rich anion intercalation properties from inorganic to organic, molecular to macromolecular and polymer anionic guests, a wide range of isotopic structures based on different stacking sequences and original bulk and surface reactivities. LDHs are under great investigation due to their interest for both fundamental and applied research fields.⁵ Indeed they have been studied for many years now as catalysts or catalyst precursors,⁷ as reinforcing charges⁸ or fire retardants⁹ for polymer development and more recently for remediation processes.¹⁰ Use of layered double hydroxides as host structures for biomolecules such as amino acids,¹¹ DNA fragments,¹² ATP¹³ or proteins¹⁴ has been developed for applications in drug storage and delivery or analytical expertise.

This paper presents the first precipitation reaction of [Zn-Al] LDHs by the use of Jack Bean urease. Urease is an enzyme which catalyses the hydrolysis of urea under mild conditions in a pH range from 4 to 9 following the equation:



The enzymatic active site is a dinuclear Ni-Ni complex located in the C chain of the monomer. The Jack Bean urease used (E C 3.5.1.5) has an activity of 54.3 units mg⁻¹ (1 unit catalyzes the formation of 1 μmol ammonia per minute at 25 °C from urea) at the optimal pH of 7.4. One monomer of Jack Bean urease has a molecular weight of 82914 Da, with a 5.4 × 9.6 nm² average size.

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Decomposition of urea can also be thermally activated at temperatures over 60 °C (the so called urea method).¹⁵ The reaction is under kinetic control and leads to the formation of basic precipitating agents. This reaction has been extensively developed for the preparation of layered double hydroxides.^{16,17} Its main interest arises from the low supersaturation conditions of precipitating reagents, which favours crystal growth over nucleation. Interestingly, this method of preparation leads to monodisperse LDH particles of large crystal dimensions of a few microns due to thermal conditions. This indeed limits their applications in processes where surface and size properties play a key role (catalysis, adsorption or separation processes, vectorisation of biomolecules).

Using urease, the decomposition of urea can take place at room temperature and the control of particle size is then possible. During this process, urease acts as both a surface with suitable electrical potential for the precipitation of LDH layers and as a templating molecule for mesopore promotion. In such conditions LDH nanosize particles may be obtained. Analogous biochemical synthesis processes were already successfully used for the precipitation of aluminium sulfate,^{18,19} calcium carbonate²⁰ or hydroxyapatite.²¹ This is the first example of the preparation of layered hydroxide materials using biomimetic conditions.

[Zn–Al] precipitation conditions were as follows: urease (5, 10 and 20 mg) was added to a 40 mL aqueous solution containing the metallic cations ($Zn^{2+} + Al^{3+} = 0.1 \text{ mol L}^{-1}$, $Zn^{2+}/Al^{3+} = 2$) and urea (urea : ($Zn^{2+} + Al^{3+}$) = 3.3, 10, 20). The suspension was aged at room temperature under N_2 and stopped at different times of between 2 to 12 days. The final product was centrifuged and washed several times with deionised water, and finally dried in air at room temperature.

The NH_4^+ concentration was measured by potentiometric titration with a selective electrode during the experiment in order to monitor the kinetics of solid formation. Its constant evolution evidences that urease was active all along the precipitation. The pH of the solution increased from 4 to 6.5 in 10 h and remained at this value until the end of the precipitation process whatever the reagent concentrations. Such a pH value is compatible with the standard coprecipitation of Zn_2Al LDHs as reported by de Roy *et al.*²²

The PXRD patterns of the materials are given in Fig. 1 for various preparation conditions.

At low urease concentration (0.1 and 0.2 g L^{-1}) and short aging time (2 days), an amorphous material is mainly obtained, which can be identified as amorphous aluminium hydroxide. When aged for a longer period of time, $Al(OH)_3$ transforms progressively into $ZnAl$ LDH phase by incorporation of Zn^{2+} cations. For higher urea concentrations, the diffractograms are characteristic of pure $ZnAl\text{-CO}_3$ hydroxalite-like compounds.²⁰ Refinement of cell parameters in the hexagonal $R\bar{3}m$ lattice gives values of $a = 0.3055$ (2) nm and $c = 2.251$ (2) nm corresponding to a basal spacing of 0.75 nm and a metal–metal distance in the layer of 0.305 nm in good agreement with a $Zn^{2+}/Al^{3+} = 2.0$. The crystallinity of the LDH strongly depends on both urea concentration and contact time with the mother solution.

Platelets of highly crystallized Zn_2Al LDH display sizes lower than 100 nm as shown by SEM and TEM experiments. EDX analysis (Fig. 2) confirms the chemical composition of the LDH for samples obtained at high urea concentration for contact time

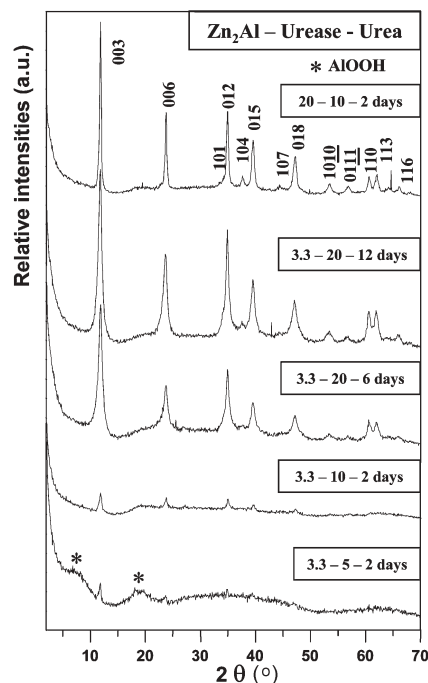


Fig. 1 PXRD diffractograms of Zn_2Al LDHs prepared by urease precipitation at different urea : ΣM^{2+} ratio—urea : urease ratio—aging time.

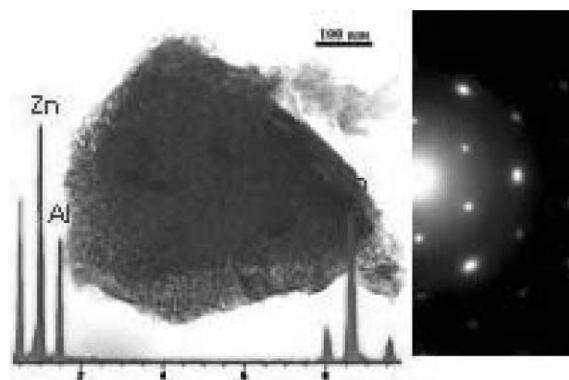


Fig. 2 TEM micrographs of Zn_2Al LDH with EDX analysis and selected area electron diffraction.

higher than 2 days. The selected area electron diffraction (SAED) displays ring-patterns in agreement with the hexagonal structure of the polycrystalline layered double hydroxides, even if in comparison, only a few theoretical inter-planar distances can be found in the electron diffraction patterns due to a preferred orientation of the platelets on the TEM grid.

SEM images (Fig. 3) show the morphology of the Zn_2Al LDH samples prepared by urease catalysed coprecipitation and the urea method. The micrograph of Zn_2AlCl prepared by thermal decomposition of urea indicates a homogeneous morphology of hexagonal-shaped primary particles (2–5 μm) forming “sand roses” aggregations where individual platelets are well identified. On the contrary, the Zn_2Al LDH samples prepared using urease, show densely packed agglomerates of particles with sizes lower than 100 nm. With aging time and increase in urea concentration,

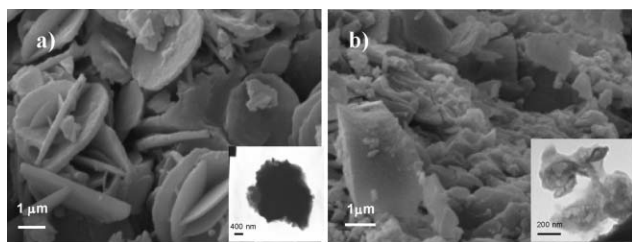


Fig. 3 SEM micrographs of Zn_2Al LDH prepared by a) urea method and b) urease catalysed precipitation.

the precipitate crumbles and the individual platelets appear with widths of over 250 nm, in agreement with the crystallinity increase. Crystal growth is expected to occur at the surface of the biomolecules allowing the control of the particle size and the formation of nanohybrid LDH-Enzyme materials. Indeed urease displays hydrophilic groups ($-NH_2$, $-OH$, $-CO_2^-$) on its external surface which can adsorb or complex Lewis acid species such as Al^{3+} cations. Nucleation of LDH must occur in the local region where metal cations accumulate and base reactants are produced *i.e.* at the surface of urease, not far from the active site. Comparison with Zn_2Al LDH prepared under thermally decomposed urea evidences strong differences in terms of crystallinity and morphology.

The FTIR spectra of the samples prepared at high urea concentrations and contact times are all characteristic²³ of hydroxalite-like compounds with typical lattice vibration bands observed in the low frequency region (ν_{M-O} : 841–647 cm^{-1} and δ_{O-M-O} : 427 cm^{-1}). The progressive formation of Zn_2Al LDH is confirmed by the increase of the δ_{O-M-O} band intensity simultaneous to the attenuation of the ν_{Al-O} band of $Al(OH)_3$ (shoulder at 1000 cm^{-1}) with increase of the aging time and urea concentration. Even though the original counter-anions of the metal salts are chloride, preferential intercalation of CO_3^{2-} is observed (ν_3 at 1360 cm^{-1}) arising from basic neutralization of decomposed urea CO_2 , due to a higher affinity of LDH structures for divalent anions.

The FTIR spectra indicate also the presence of urease entrapped at the grain boundaries. The bands of the enzyme²⁴ observed at 1652 and 1543 cm^{-1} correspond to the stretching and deformation mode of C=O (amide I) and N–H (amide II), respectively. Indeed, quantitative analysis by UV spectroscopy (absorbance measurements at $\lambda = 275$ nm) indicates that up to 90% of the urease can be immobilized by this method into Zn_2Al LDH material. Adsorption isotherms of urease by Zn_2Al LDH were measured and fitted using the Freundlich equation, indicative of strong enzyme–LDH interactions. Zn_2AlCl LDH displays a high adsorption capacity of 315 $mg\ g^{-1}$ of solid. Adsorption of the enzyme proceeds at the surface of platelets under an anion exchange mechanism. Urease is negatively charged (*i.e.* $p = 5.1$) under adsorption and preparation conditions and interacts easily *via* surface aspartate and glutamate residues and polar groups.

The novel route of LDH preparation by precipitation using urease, presented in this paper, offers a new pathway for the

control of LDH material morphology compared to standard methods. Preliminary results have demonstrated the ability to prepare $ZnAl$ LDHs with variable $Zn^{2+} : Al^{3+}$ molar ratio and Mg_2Al , Ni_2Al and Co_2Al LDHs by this novel route. It is also a way to immobilize urease in LDH. We have recently shown that urease immobilized in $ZnAl$ LDH by coprecipitation may retain up to 60% of its enzymatic activity, allowing elaboration of urea biosensors with good performances.²⁵ Current experiments are in progress to deposit thin films of these new nanohybrid urease– $ZnAl$ LDH materials and evaluate their electrical responses as biosensors for urea.

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